PHYSIOLOGIA PLANTARUM 86: 503–511. Copenhagen 1992

The state of water in acclimating vegetative buds from *Malus* and *Amelanchier* and its relationship to winter hardiness

Christina W. Vertucci and Cecil Stushnoff

Vertucci, C. W. and Stushnoff, C. 1992. The state of water in acclimating vegetative buds from *Malus* and *Amelanchier* and its relationship to winter hardiness. – Physiol. Plant. 86: 503–511.

The relationship between freezable water and cold hardiness during acclimation was studied using vegetative buds from several apple (*Malus domestica* Borkh) cultivars and from one saskatoonberry (*Amelanchier alnifolia* Nutt. cv. Smoky) cultivar. According to leakage data and visual assessments of cortical browning, vegetative buds of all cultivars were most tolerant to subfreezing temperatures in January. The hardy condition was also associated with maximum tolerance to desiccation. Qualitative features of freezing exotherms (number of peaks and temperature of the transition) were not correlated with the hardy condition in the tissues. However, the amount of unfrozen water, determined by quantifying the energy of the exotherms, increased with increasing hardiness. In buds that survived exposure to −45°C, freezing reduced the intracellular water content, but only to levels above the critical moisture content for desiccation damage. In buds that did not survive exposure to −45°C, freezing reduced the water content to levels equal to or less than the critical moisture content for desiccation damage. These observations suggest that the freezing of water in nonhardy tissue dried the tissue to moisture levels at which severe dehydration damage occurred. It appears that acclimation of vegetative apple buds involves at least two processes: (1) an increase in tolerance to dehydration and (2) an increase in the level of unfreezable water.

Key words – Acclimation, *Amelanchier*, bound water, cold hardiness, desiccation tolerance, differential scanning calorimetry, freezable water, freezing injury, *Malus*, vegetative buds.

C. W. Vertucci (corresponding author), USDA-ARS, National Seed Storage Laboratory, Fort Collins, CO 80523, USA; C. Stushnoff, Dept of Horticulture and Biochemistry, Colorado State Univ., Fort Collins, CO 80523, USA.

Introduction

Woody plant tissues become tolerant to low temperatures by preventing intracellular ice formation. Some floral and xylem tissues exhibit deep supercooling where the lack of intracellular nucleation inhibits the equilibrium condition which favors ice (Burke and Stushnoff 1979, George and Burke 1977, Quamme et al. 1972). Tissues exhibiting this behavior have limited hardiness and generally cannot survive much below the temperature of homogenous nucleation of water (−38°C) (Burke et al. 1976, George et al. 1974, Levitt 1980). In bark and vegetative buds, ice forms extracellularly and intracellular water migrates outside the cell, according to the water potential gradient, and then freezes (Burke et al. 1976, Levitt 1980). Formation of extracellular ice is associated with shrinkage of the cell (Ashworth et al. 1988), and it is believed that hardiness is limited by the tolerance of the tissues to desiccation (Burke et al. 1974, 1976, Gusta et al. 1975, Levitt 1980). More recently, it has been demonstrated that intracellular water in tissues of very hardy species forms stable glasses (Hirsh et al. 1985). In these cases, hardiness is probably dependent on the stability of the glass. Given a stable vitrified system, tissues could theoretically survive any low temperature stress.

None of the described strategies is mutually exclusive. For example, formation of extracellular ice in-

Received 8 October, 1991; revised 14 August, 1992

creases the intracellular solute concentration. The concomitant depression of the equilibrium freezing temperature of cytoplasmic water makes the intracellular solution closer to its equilibrium state at subzero temperatures. Extracellular ice formation may also allow for a sufficient concentration of solutes so that the intracellular water becomes unfreezable. It has been suggested that unfreezable water has amorphous qualities and is essentially a glass with a high melting temperature (Vertucci 1990, Williams and Leopold 1989). In other words, the formation of extracellular ice can encourage vitrification of the remaining solution in the cells. We suggest that the strategies of extracellular ice formation and vitrification are identical. Thus, the degree of hardiness may be determined by the relationship between the desiccation tolerance of the tissue and how much desiccation is required to obtain a stable glass.

Studies of the hardiness of apple twigs showed no low temperature exotherm in bark tissues (Quamme et al. 1972). However, cells shrank upon exposure to subzero temperatures (Ashworth et al. 1988), suggesting that extracellular ice formed and the protoplasm became severely desiccated. Buds from most cultivars of apple tested survived exposure to liquid nitrogen temperatures with proper treatment (Stushnoff 1987, Tyler and Stushnoff 1988). Tolerance to liquid nitrogen temperatures could be accomplished if (1) the buds were fully acclimated, (2) they were cooled slowly to −30°C and stored for up to 24 h and (3) they were then cooled rapidly to −196°C (Sakai 1965, Stushnoff 1987, Tyler and Stushnoff 1988). Controlled desiccation was also required for the cryopreservation of less hardy cultivars (Stushnoff 1987, Tyler and Stushnoff 1988). Desiccation prior to freezing treatments was not required for survival of extremely hardy tissues (Guy et al. 1986, Stushnoff 1987, Tyler and Stushnoff 1988). Studies have shown that acclimated tissues had lower water contents or higher solute concentrations (Burke and Stushnoff 1979, Burke et al. 1974, Gusta et al. 1975, Li and Weiser 1971, Sakai 1965, Tyler and Stushnoff 1988), and that slow cooling to −30°C encouraged extracellular ice formation and further reduced the amount of liquid water within cells (Sakai 1965, Stushnoff 1987, Tyler and Stushnoff 1988, Tyler et al. 1988). These studies concluded that only a certain amount of liquid water [as measured by free induction decay of nuclear magnetic resonance (NMR) signals] is allowable if tissues are to tolerate extremely low temperatures (Chen et al. 1984, Li and Weiser 1971, Stushnoff 1987, Tyler and Stushnoff 1988, Tyler et al. 1988). The second cooling process whereby tissues are rapidly cooled from −30°C to −196°C is believed to have two functions: (1) to prevent further desiccation of the cell by extracellular ice growth and (2) to encourage vitrification thereby preventing intracellular ice formation. (Hirsh et al. 1985, Sakai 1965, Stushnoff 1987, Tyler and Stushnoff 1988, Tyler et al. 1988).

There appears to be a balance between the necessity for desiccation and a sensitivity to desiccation. The present study was designed to explore the interaction between desiccation sensitivity and the presence of unfreezable water in acclimating vegetative buds from apple and *Amelanchier* twigs at different stages of acclimation to cold.

Abbreviations – DSC, differential scanning calorimetry; g g^{-1}, g H_2O (g dry weight)$^{-1}$; L_T, liquid water at temperature (T); MC, moisture content; ΔH, enthalpy.

Materials and methods

Plant tissues

Twigs from apple (*Malus domestica* Borkh) and saskatoon (*Amelanchier alnifolia* Nutt.) cultivars with varying degrees of hardiness were harvested in the fall and winter of 1987/88. Apple twigs were harvested from orchards in Fort Collins, CO (cvs Dolgo and Manchurian Crab) or Grand Junction, CO (cvs Golden Delicious and Red Delicious) and *Amelancier* (cv. Smoky) was harvested in Saskatoon, Saskatchewan. Twigs were sealed in plastic containers containing water-soaked blotter paper and stored at −5°C until used (usually within 2 weeks). Lateral buds were excised from the twigs just prior to use. Care was taken to cut buds so that xylem tissue was not included in the bud tissue studied.

Viability assays

To determine the hardiness of vegetative buds harvested between November and March, twig sections and excised buds of the cultivars were cooled at 1°C min^{-1} to temperatures as low as −50°C. [This cooling rate, although not physiological, was used because it enabled us to compare the viability of buds that were treated in the identical manner as buds used for differential scanning calorimetry (DSC) studies]. Cooling of excised buds was accomplished by loading samples into both compartments of a DSC4 (Perkin Elmer, Norfolk, CT, USA) and using a programmed cooling rate. Twig sections were sealed in plastic cryovials and cooled at 1°C min^{-1} in a methanol bath. After the cooling treatment, tissues were immediately removed and reached room temperature within 1 min. Stem sections were then placed in a Petri plate with moistened paper and allowed to incubate at 10°C for 3 weeks. After this period, browning of the cortex was evaluated on a visual rating system of 1 to 5, 1 being no cortical browning and 5 being very brown. Assessment of damage to excised buds by exposure to freezing temperatures was evaluated by using electrolyte leakage assays (Murray et al. 1990). Leakage of individual buds was measured at 10-min intervals with a 100-welled ASAC-1000 conductivity meter (Neogen, Lansing, MI, USA). The rate of leakage within the first hour of soaking was calculated and normalized to the dry weight of the bud.

Tolerance to desiccation was studied by drying buds in an open container at −5°C to various moisture levels. As the relative humidity of the refrigerator was maintained at 30%, moisture contents ranging from 0.1 to about 2.0 g H_2O (g dry weight)$^{-1}$ (g g^{-1}) could be achieved by manipulating the time of exposure to the air. Electrolyte leakage was also used as a viability assay in these experiments (Vertucci and Leopold 1987). Before leakage measurements were taken, desiccated samples were hydrated overnight at room temperature (about 22°C) and 100% relative humidity to avoid damage from imbibitional injury. The critical moisture content (MC) below which buds could not be dried without damage was determined from the point of intersection of two lines drawn for buds with higher and lower MCs. The points used in the regression analyses for buds with lower MCs had standard deviations of leakage values that were significantly greater than the standard deviation of leakage rates calculated from buds with MCs greater than 0.6 g g^{-1} (November and January harvests) or 0.8 g g^{-1} (March harvest).

Calorimetry and moisture content determinations

The thermal behavior of water in excised buds at subzero temperatures was measured by using the DSC, calibrated between 156° and −95°C with indium and methylene chloride standards. Tissue samples were hermetically sealed in aluminum pans and cooled at 1°C min^{-1}. Cooling thermograms were recorded between 0° and −60°C. There were no qualitative or quantitative differences between thermograms for apple buds cooled at this rate and at 0.2°C min^{-1}, the lower limit for scanning rate in our DSC (unpublished data). After the scan, the dry weight of the bud was determined after drying for 5 days at 75°C. Thermograms of buds with high MCs had slightly curving baselines after the exothermic event. While this may be a result of instrument artifact, it is more likely a result of small amounts of water freezing continuously after the main exotherm. To avoid bias in interpreting these thermograms, the energy of the freeze was calculated by integrating between the temperature of the onset of the exotherm and −32°C, rather than integrating only obvious exothermic events. The glass transition temperature for concentrated sucrose/water mixtures is about −32°C, so at this temperature the freezing process is theoretically complete (Hatley et al. 1991). This treatment of the data had no effect on the calculated area of exotherms from buds with MCs less than about 0.6 g g^{-1}, but tended to increase the calculated energy of exotherms from buds with higher MCs.

To determine the amount of water that did not freeze, the energies of the exotherms from buds at various moisture levels were quantified and related to the MC of the bud by least-squares fit to a linear regression. The slope of the line represents the apparent energy evolved from the freeze on a per gram of water basis, and the x-intercept is a measure of the amount of water that did not freeze (Ladbrooke and Chapman 1969, Roos 1987, Vertucci 1989, 1990). It is acknowledged that quantification of the energy from warming thermograms is preferred for a heat-compensated calorimeter such as that produced by Perkin Elmer. However, previous work (Vertucci 1990) has demonstrated a linear relationship between the energy of exotherms and the water content.

Results

From previous field experience, it is known that cvs Smoky and Dolgo are more resistant to cold injury than the other specimens studied. Cultivars Red Delicious and Golden Delicious are relatively cold-tender cultivars, and cv. Manchurian Crab has intermediate hardiness. Leakage measurements (Fig. 1) showed no differences between buds harvested in November and January except for cv. Golden Delicious. Visual ratings of

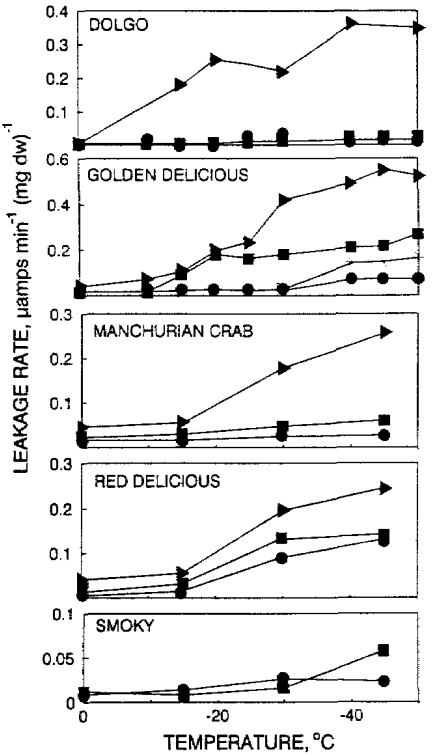

Fig. 1. The sensitivity of vegetative buds from apple and saskatoon twigs to subfreezing temperatures, as indicated by electrolyte leakage. Buds were harvested in November (squares), December (stars, cv. Golden Delicious only), January (circles) and March (triangles) and cooled at 1°C min^{-1} to various temperatures. The points represent the mean of 4 replicates. The SD for buds exposed to −15°C and above was between 0.01 and 0.04. For buds exposed to −25°C and lower, SD averaged 0.06 (November), 0.02 (January) and 0.13 (March).

Tab. 1. Visual ratings of cortical browning in stem sections of 4 apple and 1 saskatoon cvs harvested in the winter months of 1987/88 and exposed to temperatures from 0° to −45°C. The cortical tissues of stems scored as 1 were pale green and those scored as 5 were brown. Numbers represent the average of 4 replicates.

Cultivar	Harvest month	Temperature (°C)			
		0	−15	−30	−45
Dolgo	Nov	1	1	1	3
	Jan	1	1	1	1
	Mar	1	1	1.5	3.5
Golden Delicious	Nov	1	1	4.5	5
	Dec	1	1	1.5	3.5
	Jan	1	1	1	3
	Mar	1	1	2.5	4.5
Manchurian Crab	Nov	1	1	2.5	5
	Jan	1	1	1	1
	Mar	1	1	1.5	5
Red Delicious	Nov	1	1	5	5
	Jan	1	1	1.5	5
	Mar	1	1	3.5	5
Smoky Saskatoon	Nov	1.5	1.5	2	2
	Jan	2	2	2	2

cortical browning (Tab. 1) showed maximum hardiness in January for all cultivars. Of the twigs harvested in November, only cvs Dolgo and Smoky survived exposure to −45°C, cv. Manchurian Crab had hardened somewhat to −30°C, and cvs Red Delicious and Golden Delicious did not survive exposure to −30°C (Tab. 1). Except for cv. Smoky (no data collected), samples harvested in March had high leakage rates after exposure to temperatures of −30°C and lower (Fig. 1), and browning scores (Tab. 1) confirmed tissue damage at −45°C.

Changes in the sensitivity to desiccation of buds harvested at different times during the winter were evaluated by comparing initial rates of electrolyte leakage from buds dried to various moisture levels (Fig. 2). For all cultivars except cv. Smoky harvested in November, leakage rate increased abruptly when buds were dried below a critical point (Fig. 2). Data on leakage rate vs MC were fitted to two lines and the MC of the intersection was assigned as a critical moisture level, below which buds were damaged by desiccation. For all the apple cultivars, the critical MC was lowest for buds harvested in January and highest for buds harvested in March (Fig. 2, Tab. 2). Buds from cv. Smoky that were harvested in November were dried to moisture levels as low as 0.07 g g^{-1} with no increase in leakage. This extreme tolerance to desiccation was not exhibited in buds harvested in January, which showed increases in leakage at 0.23 g g^{-1}.

The freezing behavior of water in vegetative buds of the samples harvested throughout the winter was assessed by using DSC. Changes in the qualitative and quantitative characteristics of the heat flow profiles were evaluated for buds dried to different moisture levels. Representative thermograms for cvs Dolgo and

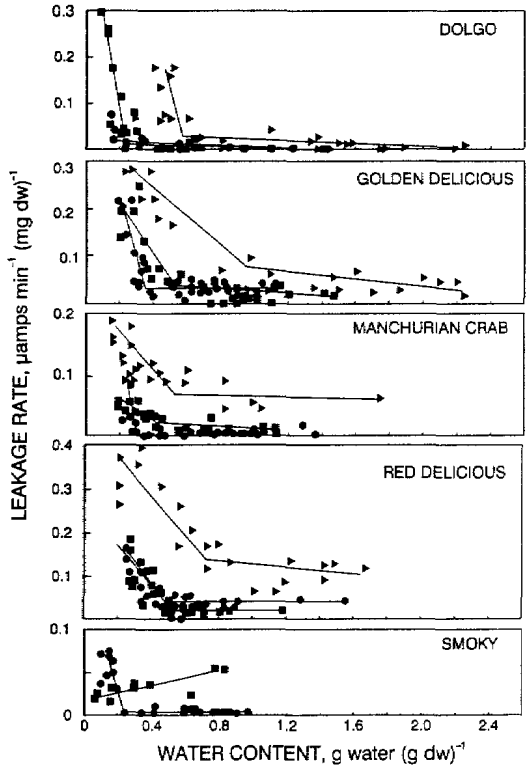

Fig. 2. The sensitivity of vegetative buds from apple and saskatoon twigs to dehydration as indicated by electrolyte leakage. Buds were harvested in November (squares), January (circles) and March (triangles), dried to different moisture levels, and then rehydrated prior to leakage measurements. The drawn curves represent the best least-squares fit of two lines to the data, except for cv. Smoky buds harvested in November, where only one line could be drawn.

Golden Delicious buds harvested in November are given in Fig. 3. There were several sharp exotherms indicating the freezing of water in hydrated buds. As the buds were dried, the exotherms became broad (Fig. 3). There were usually 2 to 4 distinct exothermic peaks in buds with moisture contents greater than 0.6 g g^{-1} regardless of the harvest date.

The temperature at which water was observed to freeze varied with MC, but not with harvest date or cultivar (Fig. 4). At MCs greater than about 0.5 g g^{-1}, the onset of freezing was between −5 and −8°C. Within this moisture range, the freezing temperature was relatively constant for buds harvested in November and January, but declined slightly with decreasing MC for buds harvested in March. For buds harvested in November or March, the onset temperature of the freeze declined sharply when buds were dried to an MC below 0.5 g g^{-1}. Freezing temperatures as low as −28°C were observed. Significant changes in freezing temperatures were not observed for buds harvested in January, be-

Tab. 2. Summary of critical MC and DSC parameters for vegetative buds of 4 apple and 1 saskatoon cultivar harvested in the winter months of 1987/88. The MC below which buds leak rapidly is the point of intersection of the lines in Fig. 2. The amount of unfrozen water, enthalpy of freezing, and coefficient of determination are calculated for the lines in Fig. 5. Superscripts a, b, and c represent coefficients that are significantly different at the level indicated in parentheses. Parameters assigned similar superscripts are not significantly different at $\alpha = 0.25$.

Cultivar	DW ± SE of bud, mg	MC below which buds leak rapidly, g H_2O (g DW)$^{-1}$	MC below which water is unfreezable, g H_2O (g DW)$^{-1}$	ΔH of freezing, $-J$ (g H_2O)$^{-1}$	Coefficient of determination, R^2
Dolgo					
Nov	2.64 ± 0.2	0.21	0.36^{b} ($\alpha = 0.01$)	290^{a}	0.97
Jan	2.73 ± 0.39	0.18	0.38^{b}	300^{a}	0.94
Mar	2.70 ± 0.20	0.58	0.26^{a}	281^{a}	0.99
Golden Delicious					
Nov	1.92 ± 0.40	0.55	0.12^{a}	201^{a}	0.91
Dec	2.30 ± 0.33	no data	0.37^{bc}	309^{b}	0.94
Jan	3.47 ± 0.36	0.38	0.40^{c} ($\alpha = 0.15$)	295^{b}	0.89
Mar	3.40 ± 0.22	0.93	0.21^{b} ($\alpha = 0.15$)	260^{b} ($\alpha = 0.05$)	0.94
Manchurian Crab					
Nov	4.31 ± 0.43	0.41	0.41^{b} ($\alpha = 0.01$)	285^{a}	0.98
Jan	2.97 ± 0.29	0.31	0.41^{b}	331^{a}	0.95
Mar	3.95 ± 0.32	0.52	0.23^{a}	280^{a}	0.99
Red Delicious					
Nov	3.28 ± 0.24	0.52	0.40^{b}	312^{a}	0.91
Jan	3.07 ± 0.51	0.47	0.39^{b} ($\alpha = 0.01$)	308^{a}	0.98
Mar	3.31 ± 0.27	0.75	0.28^{a}	292^{a}	0.98
Smoky Saskatoon					
Nov	4.67 ± 0.29	not detected	0.30^{a}	288^{a}	0.99
Jan	2.18 ± 0.12	0.23	0.30^{a}	311^{a}	0.99

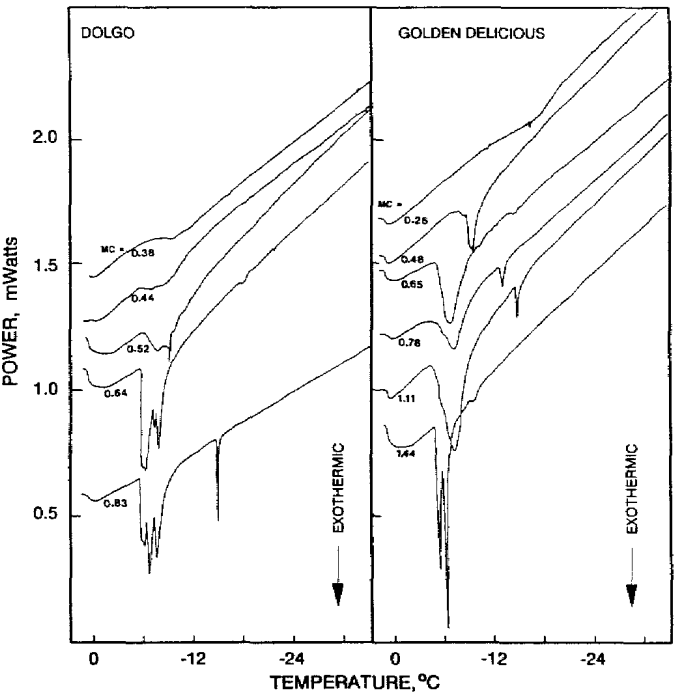

Fig. 3. Heat flow profiles of cvs Dolgo and Golden Delicious buds harvested in November 1987 and dried to indicated water levels (on a g g^{-1} basis). Heat flow profiles were recorded as buds were cooled at 1°C min^{-1}.

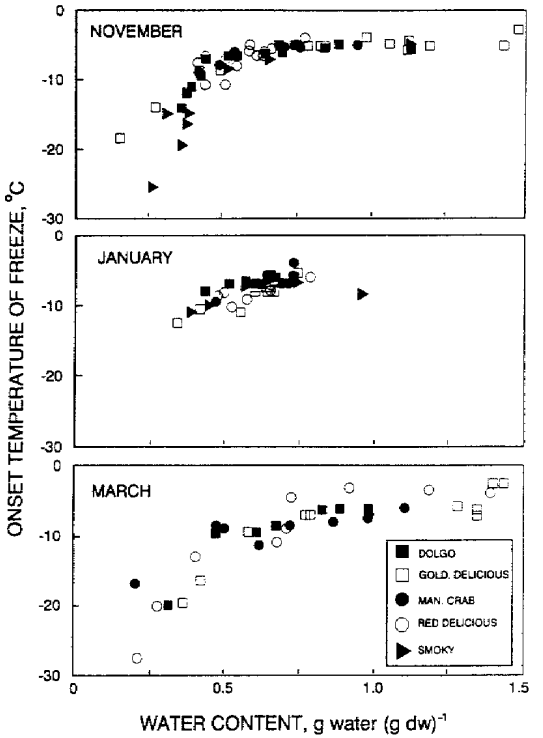

Fig. 4. The onset temperature of water freezing in vegetative buds from apple and saskatoon harvested and dried in November, January and March. Symbols are for different cultivars as indicated in the legend. Data were taken from heat flow profiles similar to those in Fig. 3.

cause MCs were generally less than 0.75 g g^{-1} and transitions were not observed in buds with MCs less than about 0.30 g g^{-1} (Fig. 4 and Tab. 2). When the MC of the sample was considered, there was no discernable trend between the number of exotherms or the onset temperature of exotherms as a function of harvest date.

The energy of the freezing transition was quantified by measuring the area of the freezing exotherms. There is a linear relationship between this quantity (ΔH), normalized to the dry weight of the bud, and the MC of the bud (Fig. 5). The amount of water which did not freeze is the x-intecept of the line. The level of unfrozen water in bud tissues ranged from 0.12 g g^{-1} to 0.41 g g^{-1} and changed during the course of the winter (Fig. 5, Tab. 2). Generally, the level of unfrozen water was ≥ 0.3 g g^{-1} for buds harvested in January and November and < 0.3 g g^{-1} for buds harvested in March. Buds harvested from cv. Golden Delicious in November are an exception, as freezing transitions can be detected in buds with MCs as low as 0.12 g g^{-1}. The slope of the ΔH (dry weight)$^{-1}$ vs MC curve reflects the energy of the freeze on a per gram of water basis. Although differences among harvest dates were not significant at $\alpha = 0.25$, the value for the energy of the freeze tended towards what is expected for pure water [−333 J (g $H_2O)^{-1}$] in midwinter (Tab. 2).

During the winter months, there is a change in the low temperature limit to which apple buds can be exposed without significant increases in leakage or cortical browning. Similarly, there is a change in the level of dehydration that the buds can withstand without increases in leakage. While the temperature at which water freezes does not change as a function of harvest date, the amount of water that remains unfrozen increases, and the energy of the water that does freeze approaches that of pure water during the winter months (Tab. 2).

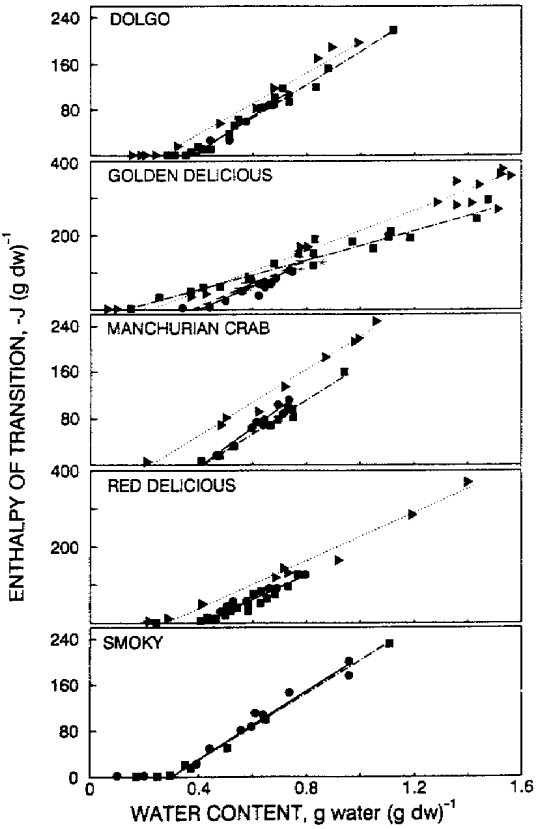

Fig. 5. The energy of the freezing transition in vegetative buds from apple and saskatoon twigs harvested in November (squares), December (stars, cv. Golden Delicious only), January (circles) and March (triangles). Buds were dried to various moisture levels to achieve the ΔH (dry weight)$^{-1}$ vs water content relationship. Transition enthalpies were determined by measuring the area of exotherms similar to those in Fig. 3. The lines are the least-squares best fit to the data.

Discussion

The experiments in this study were designed to determine the interaction between hardiness level, tolerance to desiccation and the freezing behavior of water in dormant vegetative buds of 4 apple and one saskatoon cultivar. Buds were most tolerant to low temperatures in January (Tab. 1, Fig. 1). Except for cv. Smoky, tolerance to desiccation followed the same trend as bud hardiness: when buds were hardier, they also tolerated dehydration to lower MCs without significant increases in leakage (Tab. 2, Fig. 2). There was a trend towards increasing levels of unfrozen water in the hardened condition: when buds of a given cultivar were hardiest they usually had the highest level of unfrozen water (Tab. 2). However, the amount of unfrozen water did not appear to be linked to hardiness per se, since the level of unfrozen water was sometimes higher in cold-tender cultivars. In cases where buds apparently survived exposure to −45°C [browning ≤ 3 (column 6, Tab. 1) and low leakage (Fig. 1)], the critical MC for desiccation damage was always lower than the MC at which only unfrozen water was present (compare columns 3 and 4, Tab. 2). In cases where tissues did not survive −45°C [browning ≥ 4 (column 6, Tab.1)] or were severely damaged by exposure (browning = 3.5 and high leakage), the critical MC for desiccation damage was equal to or greater than the level of unfrozen water (Tab. 2).

It has been hypothesized that extracellular freezing may remove "vital water" from the cell resulting in a lethal desiccation stress, and that hardiness is dependent on the water-retaining power of the cell (Tumanov 1969, Weiser 1970). This hypothesis has not received strong support from studies of the amount of unfreezable water in tissues in relation to their level of acclimation. While some researchers report increases of "bound" water with acclimation (Chen et al. 1984, Johansson 1970, Krasavetsev 1968, Samygin and Livshin 1970, Storey 1983, Storey and Storey 1983, Tumanov 1969), others have shown that there is no relationship between unfreezable water and hardiness (Burke et al. 1974, George and Burke 1977, Gusta et al. 1975, Tyler et al. 1988). Methods of quantification of the amount of unfreezable water may have contributed to the controversy. In some calorimetric studies, the amount of unfreezable water was calculated assuming that the heat of fusion of water in concentrated solutions is identical to that of pure water [−333 J $(g\ H_2O)^{-1}$] (Johansson 1970, Krasavetsev 1968, Samygin and Livshin 1970, Tumanov 1969). Recent studies have demonstrated that this approach may be erroneous (Blond 1989, Hatley et al. 1991, Vertucci 1990). The calculations of the level of unfrozen water in the present study do not rely on an assumed heat of fusion of pure water. However, there are limitations to this method in that determinations of the baseline from which area measurements are made are difficult. Also, because the temperature of freezing varied with moisture content and the heat of fusion is a nonlinear function of temperature, we would expect some nonlinear behaviour of the enthalpy vs moisture content relationships.

Studies calculating the amount of unfreezable water by using NMR are also subject to interpretation because of the nonlinear nature of L_T vs T^{-1} plots (George and Burke 1977, Gusta et al. 1975, Tyler et al. 1988). In most cases, L_T deviates strongly from a linear relationship with T^{-1} at about −30°C. Thus, calculations of the level of unfrozen water will be affected by the temperature range of the linear regression data as well as the initial moisture content of the tissue (the latter factor affects the temperature at which water freezes). In spite of these difficulties, slight increases in the level of unfrozen water were reported for cereals with increasing hardiness within a cultivar, but there was no correlation between cultivars (Gusta et al. 1975).

NMR studies of the amount of liquid water remaining after equilibration at subzero temperatures have demonstrated that (1) the amount of water that freezes is generally not correlated with tolerance to low temperatures and (2) the amount of water that remains liquid is not related to hardiness. Our results are consistent with the first finding, and can be justified by rationalizing that, given fully hardy tissue, more water will freeze in a hydrated sample (because there is more water) than in a slightly desiccated sample, and yet both tissues can survive exposure to −45°C. This is the principle behind the linear plots of ΔH vs MC in Fig. 5. Our results do not support the second conclusion of the NMR studies, mostly because of interpretation. NMR measures the mobility of protons. The line widths for fluid aqueous solutions range from several Hz to about 8×10^3 Hz; protons giving these line widths are said to be from "liquid" water (Burke et al. 1974). However, water at macromolecular interfaces has restricted mobility, and though it is liquid in the strictest sense, it may give NMR line widths of between 3×10^4 and 10^5 Hz. It is believed that this type of water has glassy tendencies and does not freeze (Vertucci 1990, Williams and Leopold 1989). It is also suggested that this type of water is important to the desiccation tolerance in plant tissues (Burke 1986, Pammenter et al. 1991, Vertucci and Leopold 1987, Williams and Leopold 1989). Because of the restricted mobility, measurement of this water at ambient temperatures by using NMR at frequencies less than 3×10^4 is difficult. Because this water does not freeze, its phase properties change only slightly when the sample is cooled to subzero temperatures (Vertucci 1990). Consequently, measurements of the amount of water that remains "liquid" (i.e. mobile enough to be detected with NMR) at −50°C do not account for the water that was sufficiently immobilized to be undetected by NMR even at room temperature. It is the latter type of water which we postulate increases during acclimation.

Evidence supporting our hypothesis that the condi-

tion of water changes with acclimation can be derived from NMR studies at room temperature, which show significantly faster relaxation times (reduced proton mobility) for water in acclimated compared to non-acclimated stems of Red Osier dogwood, especially in the signals considered to be intracellular water (Burke et al. 1974). Based on NMR images of dormant and nondormant vegetative apple buds, it has been postulated that the amount of "bound" water changes during the winter months and that water in dormant buds has restricted mobility (Faust et al. 1991).

It has been suggested that survival at low temperatures is a function of tolerance of, rather than resistance to, desiccation (Burke et al. 1974, 1976, George et al. 1974, Gusta et al. 1975, Levitt 1980). Our data suggest that freezing tolerance in vegetative buds is a function of both the ability to survive dehydration and the ability to maintain a certain level of water. Both the level of desiccation tolerance and the amount of unfreezable water change during acclimation. We suggest that hardiness results when the freezing of water does not desiccate tissues to damaging levels; this is achieved when the level of unfrozen water is greater than the critical moisture level for desiccation damage.

Comparisons of the apparent energy of water freezing [slopes of ΔH (dry weight)$^{-1}$ vs MC curves in Fig. 5] suggest that this value changed in samples harvested at different times and was most similar to pure water in buds harvested in January. Since the calculated heat of fusion is a composite of several processes, including the heat of fusion of pure water, ice adsorption, the disassociation of solutes and macromolecular surfaces from solvent water and the temperature dependence of the enthalpy of fusion (Burke et al. 1974, Hatley et al. 1991, Olien 1974, Vertucci 1990), it would be expected that the measured values would be less than the theoretical value of -333 J (g H_2O)$^{-1}$. It is suggested that the low enthalpies calculated for cold-tender buds may be a result of artifacts induced by the rapid cooling rates (1°C min^{-1}) and quantification of the energy in exotherms: it is conceivable that water which would ordinarily migrate to the extracellular spaces is not given adequate time to do so at moderately low temperatures, and so is not detected.

It is generally accepted that the limit of tolerance to low temperatures for woody tissues exhibiting extracellular ice formation is a function of the desiccation tolerance of the tissue. The results presented in the present paper are consistent with this hypothesis. Our results also indicate that the ability to reduce the level of desiccation by increasing the level of unfrozen water is augmented in hardy tissues. Thus, we conclude that hardiness to low temperatures is achieved in a two-step process whereby both resistance and tolerance to desiccation increase. The process may involve changes in the concentration of protective solutes or in the behavior of water at macromolecular interfaces.

Acknowledgments – The authors would like to thank Dr R. Renquist (Orchard Mesa Research Center, Dept of Horticulture, Colorado State Univ.) for providing apple twigs from Grand Junction, CO, and R. Sawatzky (Dept of Horticulture, Univ. of Saskatchewan) for providing saskatoon twigs from Saskatoon, Saskatchewan. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Dept of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may be suitable.

References

Ashworth, E.N., Echlin, P., Pearce, R.S. & Hayes, T.L. 1988. Ice formation and tissue response in apple twigs. – Plant Cell Environ. 11: 703–710.

Blond, G. 1989. Water-galactose system: supplemented state diagram and unfrozen water. – Cryo. Lett. 10: 299–308.

Burke, M.J. 1986. The glassy state and survival of anhydrous biological systems. – *In* Membranes, Metabolism and Dry Organisms (A.C. Leopold, ed.), pp. 358–363. Cornell University Press, Ithaca, NY. ISBN 0-8014-1979-4.

– & Stushnoff, C. 1979. Frost hardiness: A discussion of possible molecular causes of injury with particular reference to deep supercooling of water. – *In* Stress Physiology in Crop Plants (H. Mussell and R.P. Staples, eds), pp. 197–225. Wiley Interscience, New York, NY. ISBN 0-471-03809-1.

– , Bryant, R.G. & Weiser, C.J. 1974. Nuclear magnetic resonance of water in cold acclimating red osier dogwood stem. – Plant Physiol. 54: 392–398.

– , Gusta, L.V., Quamme, H.A., Weiser, C.J. & Li, P.H. 1976. Freezing and injury in plants. – Annu. Rev. Plant Physiol. 27: 507–528.

Chen, T.H., Kartha, K.K., Constabel, F. & Gusta, L.V. 1984. Freezing characteristics of cultured *Catharanthus roseus* (L). G. Don cells treated with dimethylsulfoxide and sorbitol in relation to cryopreservation. – Plant Physiol. 75: 720–725.

Faust, M., Liu, D., Millard, M.M. & Stutte, G.W. 1991. Bound versus free water in dormant apple buds – a theory for endodormancy. – HortScience 26: 887–890.

George, M.F. & Burke, M.J. 1977. Cold hardiness and deep supercooling in xylem of shagbark hickory. – Plant Physiol. 59: 319–325.

– , Burke, M.J., Pellett, H.M. & Johnson, A.G. 1974. Low temperature exotherms and woody plant distribution. – HortScience 9: 519–522.

Gusta, L.V., Burke, M.J. & Kapoor, A.C. 1975. Determination of unfrozen water in winter cereals at subfreezing temperatures. – Plant Physiol. 56: 707–709.

Guy, C.L., Niemi, K.J., Fennell, A. & Carter, J.V. 1986. Survival of *Cornus sericea* L. stem cortical cells following immersion in liquid helium. – Plant Cell Environ. 9: 447–450.

Hatley, R.H.M., van den Berg, C. & Franks, F. 1991. The unfrozen water content of maximally freeze concentrated carbohydrate solutions: validity of the methods used for its determination. – Cryo. Lett. 12: 113–124.

Hirsh, A.G., Williams, R.J. & Meryman, H.T. 1985. A novel method of natural cryoprotection. – Plant Physiol. 66: 40–45.

Johansson, N.O. 1970. Ice formation and frost hardiness in some agricultural plants. – Nat. Swed. Inst. Plant Protection Contrib. 14: 364–382.

Krasavetsev, O.H. 1968. Amount of nonfrozen water in woody plants at different temperatures. – Fiziol. Rast. 15: 225–235.

Ladbrooke, B.D. & Chapman, D. 1969. Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent studies. – Chem. Phys. Lipids 3: 304–356.

Levitt, J. 1980. Responses of Plants to Environmental Stress, Vol. 1. Chilling Freezing and High Temperature Stresses. – Academic Press, New York, NY. ISBN 0-12-445501-8.
Li, P.H. & Weiser, C.J. 1971. Increasing cold resistance of stem sections of *Cornus stolonifera* by artificial dehydration. – Cryobiology 8: 108–111.
Murray, M.B., Cape, J.N. & Fowler, D. 1990. Quantification of frost damage in plant tissues by rates of electrolyte leakage. – New Phytol. 113: 307–311.
Olien, C.R. 1974. Energies of freezing and frost desiccation. – Plant Physiol. 53: 764–767.
Pammenter, N.W., Vertucci, C.W. & Berjak, P. 1991. Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. – Plant Physiol. 96: 1093–1098.
Quamme, H., Stushnoff, C., & Weiser, C.J. 1972. The relationship of exotherms to cold injury in apple stem tissues. – J. Am. Soc. Hortic Sci. 97: 608–613.
Roos, Y.H. 1987. Effect of moisture on the thermal behavior of strawberries studied using differential scanning calorimetry. – J Food Sci. 52: 146–149.
Sakai, A. 1965. Survival of plant tissue at super-low temperatures. III. Relation between effective prefreezing temperatures and the degree of frost hardiness. – Plant Physiol. 40: 882–887.
Samygin, G.A. & Livshin, A.Z. 1970. Water retaining forces in the cells of various plants in relation to their resistance to dehydration and freezing during the formation of extracellular ice. – Sov. Plant Physiol. 72: 114–119.
Storey, K.B. 1983. Metabolism and bound water in overwintering insects. – Cryobiology 2: 365–379.
– & Storey, J.M. 1983. Biochemistry of freeze tolerance in terrestrial insects. – Trends Biochem. Sci. 8: 242–245.
Stushnoff, C. 1987. Cryopreservation of apple genetic resources. – Can. J. Plant Sci. 67: 1151–1154.
Tumanov, I.I. 1969. Physiology of plants not killed by frost. – Izv. Acad. Nauk, USSR Ser. Biol. 4: 469–480.
Tyler, N.J. & Stushnoff, C. 1988. The effects of prefreezing and controlled dehydration on cryopreservation of dormant vegetative apple buds. – Can. J. Plant Sci. 68: 1163–1167.
–, Stushnoff, C. & Gusta, L.V. 1988. Freezing of water in dormant vegetative apple buds in relation to cryopreservation. – Plant Physiol. 87: 201–205.
Vertucci, C.W. 1989. Relationship between thermal transitions and freezing injury in pea and soybean seeds. – Plant Physiol. 90: 1121–1128.
– 1990. Calorimetric studies of the state of water in seed tissues. – Biophys. J. 58: 1463–1471.
– & Leopold, A.C. 1987. The relationship between water binding and desiccation tolerance in tissues. – Plant Physiol. 85: 232–238.
Weiser, C.J. 1970. Cold resistance and injury in woody plants. – Science 169: 1269–1278.
Williams, R.J. & Leopold, A.C. 1989. The glassy state in corn embryos. – Plant Physiol. 89: 977–981.

Edited by T. C. Hsiao